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Published in:
Developmental Biology

DOI:
10.1016/0012-1606(82)90087-2

Publication date:
1982

DEVELOPMENTAL BIOLOGY **94**, 240–245 (1982)

Phosphodiesterase Induction in *Dictyostelium discoideum* by Inhibition of Extracellular Phosphodiesterase Activity

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Received May 3, 1982; accepted July 15, 1982

Adenosine 3',5'-monophosphate (cAMP) is a chemoattractant in *Dictyostelium discoideum*; it also induces phosphodiesterase activity. Recently it was shown (M. H. Juliani, J. Brusca, and C. Klein, (1981) *Develop. Biol.* **83**, 114–121) that N^6-(aminohexyl)adenosine 3',5'-monophosphate (hexyl-cAMP) effectively induced phosphodiesterase activity, while this compound was chemotactically inactive and did not effectively bind to the cell surface receptor for cAMP. It was suggested that hexyl-cAMP and cAMP induce phosphodiesterase activity via a chemoreceptor-independent mechanism. In another recent report (P. J. M. Van Haastert, R. C. Van der Meer, and T. M. Konijn (1981) *J. Bacteriol.* **147**, 170–175) investigation of induction of phosphodiesterase by several cAMP derivatives revealed that phosphodiesterase induction and chemotaxis had similar cyclic nucleotide specificity. Based on this result it was suggested that cAMP induces phosphodiesterase activity via activation of the chemotactic receptor. In this report we show that hexyl-cAMP transiently inhibits extracellular and cell surface phosphodiesterase. This transient inhibition of the inactivating enzyme and the permanent release of small amounts of cAMP by the cells leads to a transient increase of extracellular cAMP levels. Hexyl-cAMP does not inhibit beef heart phosphodiesterase, and is not degraded by this enzyme. Addition of hexyl-cAMP to a cell suspension containing beef heart phosphodiesterase does not result in an accumulation of extracellular cAMP, and phosphodiesterase induction is absent. We conclude that hexyl-cAMP inhibits phosphodiesterase activity which leads to the accumulation of cAMP; consequently cAMP binds to the chemotactic cAMP receptor resulting in the induction of phosphodiesterase activity.

INTRODUCTION

In the presence of nutrients the cellular slime mold *Dictyostelium discoideum* grows as single cells. When the food supply is exhausted, the cells pass a transient phase, after which cell aggregation starts, followed by the formation of a fruiting body. Cell aggregation is mediated by chemotaxis to cAMP (Konijn *et al.*, 1967), which is secreted by neighboring cells (Gerisch and Wick, 1975; Roos *et al.*, 1975; Shaffer, 1975). Extracellular cyclic AMP is hydrolyzed by extracellular or membrane-bound phosphodiesterase (PDE; EC 3.1.4.17) (Pannbacker and Bravard, 1972; Malchow *et al.*, 1972). Extracellular cAMP may also bind to nonhydrolyzing cell surface receptors for cAMP (Malchow and Gerisch, 1974; Green and Newell, 1975; Henderson, 1975; Mato and Konijn, 1975); this may ultimately lead to pseudopod formation.

cAMP is not only involved in chemotaxis during cell aggregation, but also in the differentiation to aggregation competence. Under some conditions (see Marin and Rothman, 1980) pulses of cAMP reduce the length of the interphase (Darmon *et al.*, 1975; Gerisch *et al.*, 1975), and induce an earlier increase of PDE activity, cAMP receptors, and contact sites A (Gerisch *et al.*, 1975; Klein and Darmon, 1975, 1977).

Recently, two reports appeared in which the cell's detection mechanism of the cAMP signal for cell differentiation and PDE-induction was investigated (Juliani *et al.*, 1981; Van Haastert *et al.*, 1981). Juliani *et al.* (1981) showed that N^6-(aminohexyl)-adenosine 3',5'-monophosphate (hexyl-cAMP) is chemotactically at least 10,000-fold less active than cAMP, and that it binds to the cell surface receptor with approximately a 10,000-fold lower affinity. In contrast to this low affinity for the chemotactic receptor only 10 times higher concentrations than cAMP were sufficient to induce cell differentiation and PDE induction. These results were explained by assuming that hexyl-cAMP (and, therefore, cAMP) influence cell differentiation by a cAMP-receptor-independent mechanism. Van Haastert *et al.* (1981) showed that the threshold concentrations of several cAMP derivatives to induce PDE activity parallel the threshold concentrations for chemotaxis. This was interpreted as evidence for the involvement of the chemotactic cAMP receptor in PDE induction. The dose–response curves of the cAMP derivatives revealed that at high concentrations a derivative with a high threshold concentration induced more PDE activity than a derivative with a lower threshold concentration. This could be explained by assuming that the cAMP receptor is a rate receptor, which means that its activity depends on the frequency of cAMP–receptor combinations, rather than on the fraction of receptors occupied by cAMP.

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0012-1606/82/110240-06$02.00/0

The effects of cAMP derivatives on cell differentiation and PDE-induction may be explained by the following mechanisms: (1) A cAMP–receptor-independent mechanism; the similar cyclic nucleotide specificities for chemotaxis and PDE induction is a coincidence. (2) Hexyl-cAMP might be a very good substrate of the extracellular and cell surface PDE, by which hexyl-cAMP transiently inhibits the hydrolysis of cAMP. This will allow the cAMP released by the cells to accumulate, and to activate the cAMP receptor. (3) Hexyl-cAMP binds to the cell surface receptor, which is a rate receptor. Association of hexyl-cAMP to the receptor is with an on-rate comparable to cAMP, but dissociation occurs with a very high off-rate. This would imply that at equimolar concentrations the frequency of hexyl-cAMP–receptor-interactions is comparable to that of cAMP, while the fraction of receptors occupied with hexyl-cAMP is much less than the fraction occupied with cAMP.

Here we report that hexyl-cAMP effectively inhibits phosphodiesterase activity resulting in the accumulation of cAMP. Hexyl-cAMP does not induce PDE activity if the release of cAMP is not allowed to accumulate. These observations question the presence of a cAMP–receptor-independent detection mechanism in *D. discoideum*.

MATERIALS AND METHODS

Materials

6-Chloropurine-riboside-3′,5′-monophosphate (6-Cl-cPRMP), 1,6-diaminohexane, and snake venom (*Ophiophagus hannah*) were obtained from Sigma; beef heart phosphodiesterase and cAMP were purchased from Boehringer; theophylline was from Merck; [8-^{3}H]cAMP (0.9 TBq/mmole) and the cAMP binding protein assay were obtained from Amersham.

N^6-(Aminohexyl)-adenosine 3′,5′-monophosphate (hexyl-cAMP) was synthesized and purified as described by Juliani *et al.* (1981). The purity was analyzed by high-performance liquid chromatography (HPLC) using different stationary phases and various mobile phase compositions to optimize the separation of nucleotides and nucleosides (Van Haastert, 1981a,b). The final preparation had a purity of about 90%; cAMP or 6-Cl-cPRMP were below the detection limit (<0.1%). The impurities were not degraded by beef heart PDE or by *D. discoideum* PDE, which suggests that they are not cyclic nucleotides.

Cell and Culture Conditions

All experiments were carried out with *Dictyostelium discoideum*, NC-4(H), which was grown in association with *Escherichia coli* B/r on solid medium containing 3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 2H_2O$, and 15 g agar per liter. Cells were harvested in the late log phase with 10 m*M* sodium-potassium phosphate buffer, pH 6.5, and freed from bacteria by repeated centrifugations. Cells were starved in phosphate buffer, pH 6.5, at a density of 10^7 cells/ml. After 1 hr cells were centrifuged, washed twice with 10 m*M* phosphate buffer, pH 7.0, and resuspended in this buffer at various densities.

Isolation of Extracellular Phosphodiesterase Activity

This enzyme was isolated from 2×10^9 cells with a slightly changed method of Orlow *et al.* (1981). After the ammonium sulfate precipitation step, the pellet was dissolved in 5 ml 10 m*M* phosphate buffer, pH 7.0, and concentrated on Minicon B15 (Amicon, Oosterhout, the Netherlands) to 0.5 ml. The concentrate was washed twice with 5 ml of this buffer, and dissolved in 1.3 ml of the same buffer. The preparation contained 2 mg protein/ml, and hydrolyzed 1500 nmole/min/mg protein at 10^{-5} *M* cAMP.

Inhibition of the Hydrolysis of [^{3}H]cAMP by cAMP, Hexyl-cAMP, and Theophylline

Extracellular phosphodiesterase. The incubation mixture (100 μl) contained 10 m*M* phosphate buffer, pH 7.0, 10^{-7} *M* [^{3}H]cAMP (approximately 1 KBq), 2 ng extracellular PDE, and various concentrations cAMP, hexyl-cAMP, or theophylline. The reaction was terminated after 30 min at 22°C by boiling the samples for 2 min. After cooling, 50 μg snake venom was added, and the sample was incubated at 37°C. After 30 min 1 ml Dowex AG1-X2 was added (one part ion exchanger and two parts water). Samples were shaken for 2 min, followed by centrifugation. The radioactivity was determined in 500 μl of the supernatant.

Cell surface PDE. The incubation mixture (100 μl) contained the same components as for extracellular PDE, except that extracellular PDE was replaced by 10^6 freshly washed cells. The reaction was terminated after 3 min at 22°C by the addition of 100 μl perchloric acid (3.5%, v/v). The lysates were neutralized with 50 μl $KHCO_3$ (50% saturated solution at 22°C) and centrifuged. The supernatant (150 μl) was incubated with snake venom and ion exchanger as described above.

Beef heart PDE. The assay was identical to the extracellular PDE assay, except that the buffer was 50 m*M* Tris/HCl, 2 m*M* $MgSO_4$, pH 7.5, that the temperaure was 30°C, and that 250 ng beef heart PDE was used.

Hydrolysis of cAMP and Hexyl-cAMP

Extracellular PDE. The incubation mixture (30 μl) contained 10 m*M* phosphate buffer, pH 7.0, 2×10^{-4} *M*

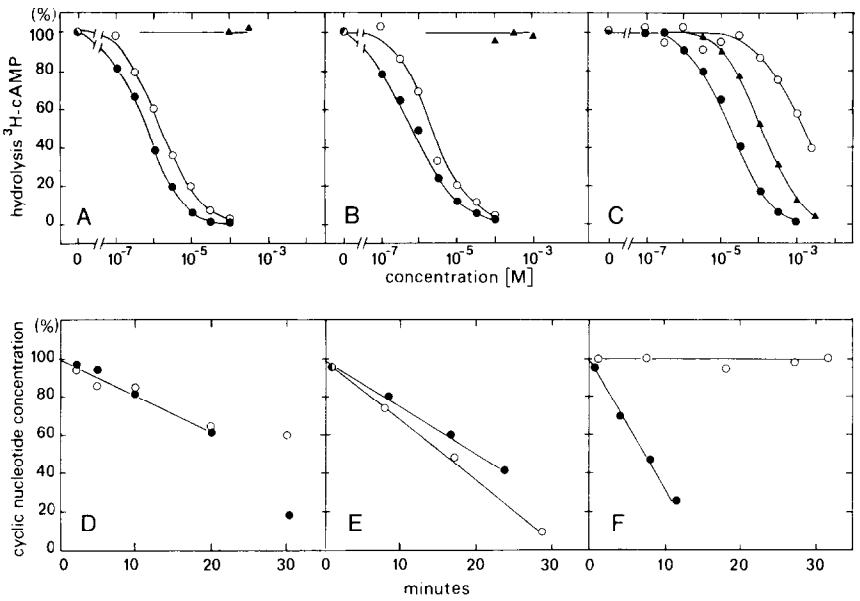

FIG. 1. Interaction of cAMP, hexyl-cAMP, and theophylline with PDE. (A, B, C) The hydrolysis of [^{3}H]cAMP was measured at 10^{-7} *M* radiolabel in the presence of various concentrations of nonradioactive cAMP (●), hexyl-cAMP (○), or theophylline (▲). (D, E, F) The hydrolysis of cAMP (●) and heyxl-cAMP (○) was measured at 2×10^{-4} *M* (D and E) or 2×10^{-3} *M* (F) by separation of the compounds of the reaction mixtures by HPLC. (A, D) Extracellular PDE of *D. discoideum*; (B, E) cell surface PDE of *D. discoideum*; (C, F) beef heart PDE.

To investigate this hypothesis we searched for a phosphodiesterase preparation which would not be inhibited by micromolar hexyl-cAMP concentrations. Addition of such an enzyme to the cell suspension should prevent the cAMP accumulation induced by hexyl-cAMP. Beef heart PDE has a high K_m for cAMP (Butcher and Sutherland, 1962), and exhibits some specificity for cAMP in the adenine moiety (Simon *et al.*, 1973). Furthermore, this enzyme is inhibited by low concentrations of theophylline (Butcher and Sutherland, 1962), while the slime mold phosphodiesterases are not inhibited by theophylline (Chang, 1968).

TABLE 2
PDE-INDUCTION (%) BY cAMP AND HEXYL-cAMP IN THE PRESENCE OF BEEF HEART PDE

Stimulus	Enzyme added: Buffer	Beef heart PDE	Boiled beef heart PDE
10^{-7} *M* cAMP	28 ± 2	29 ± 6	34 ± 8
10^{-6} *M* Hexyl-cAMP	11 ± 7	−3 ± 4	12 ± 6
10^{-5} *M* Hexyl-cAMP	67 ± 8	4 ± 6	65 ± 10

Note. Buffer, beef heart PDE, or boiled beef heart PDE were added to a suspension of postvegetative *D. discoideum* cells. These suspensions were pulsed at 5-min intervals for 1 hr with buffer, 10^{-7} *M* cAMP, 10^{-6} *M* hexyl-cAMP, or 10^{-5} *M* hexyl-cAMP (final concentrations). After homogenation samples were tested for slime mold PDE activity by including 1 m*M* theophylline in the assay mixture. PDE induction is presented as the % increase of PDE activity over the activity of suspensions pulsed with buffer. Suspensions were pulsed in duplicates; PDE activity was assayed in triplicates. The means and standard deviations are shown.

Figure 1C shows that beef heart PDE is inhibited only by millimolar concentrations of hexyl-cAMP; in addition, hydrolysis of hexyl-cAMP at high substrate concentrations has not been observed (Fig. 1F), indicating an at least 10-fold smaller V_{max} for hexyl-cAMP if compared to cAMP. Furthermore, 1 m*M* theophylline inhibits at least 90% of the beef heart PDE, while the slime mold PDEs are inhibited by less than 5% if 1 m*M* theophylline is present. Thus, by addition of 1 m*M* theophylline to a mixture of slime mold PDE and beef heart PDE only the hydrolysis by slime mold PDE will be detected.

PDE induction by hexyl-cAMP in the presence of beef heart PDE. The amount of beef heart PDE which was added to the cell suspension expressed about the same activity at 10^{-7} *M* cAMP as the cell suspension without added PDE. As a control, the same amount of boiled beef heart PDE was added to the cell suspension. The activity of unboiled beef heart PDE in a cell suspension of *D. discoideum* remains constant during at least 1 hr. This was assayed with 3′-deoxy, 3′-aminoadenosine 3′,5′-monophosphate (3′-NH-cAMP), which is a good substrate of the beef heart PDE, but which is not hydrolyzed by the slime mold PDE (data not shown).

The results of Table 2 reveal that boiled beef heart PDE has no effect on the induction of slime mold PDE by 10^{-7} *M* cAMP, or by 10^{-6} or 10^{-5} *M* hexyl-cAMP.

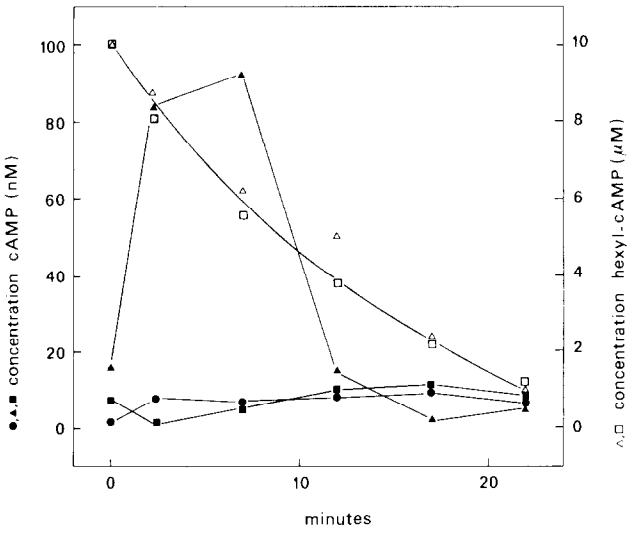

FIG. 2. Accumulation of extracellular cAMP in the presence of hexyl-cAMP. Postvegetative *D. discoideum* cells were incubated with or without 10^{-5} *M* hexyl-cAMP in the presence or absence of beef heart phosphodiesterase. At the times indicated samples were withdrawn and assayed for cAMP or hexyl-cAMP (see Materials and Methods). (●) Incubation without hexyl-cAMP and without beef heart PDE; (▲, △) incubation with hexyl-cAMP and without beef heart PDE; (■, □) incubation with hexyl-cAMP and with beef heart PDE. (●, ▲, ■) Concentration of cAMP; (△, □), concentration of hexyl-cAMP. The experiment was repeated twice yielding the same results.

Active beef heart PDE also has no effect on the induction of slime mold PDE by 10^{-7} *M* cAMP, indicating that the detection mechanism of cAMP is still intact. In contrast, hexyl-cAMP is not able to induce slime mold PDE in the presence of beef heart PDE.

Hexyl-cAMP Induces a Temporal Accumulation of Extracellular cAMP

Figure 2 shows that addition of hexyl-cAMP to a cell suspension results in a temporal accumulation of extracellular cAMP. This accumulation does not take place in the absence of hexyl-cAMP, or if hexyl-cAMP and beef heart phosphodiesterase are added simultaneously. In these experiments hexyl cAMP was separated from cAMP by ion-exchange chromatography, and cAMP levels were measured with a binding protein assay.

Phosphodiesterase Induction by other cAMP Derivatives

The foregoing results indicate that hexyl-cAMP induces PDE-activity by protection of cAMP released by the cells against degradation. This protection is due to the high affinity of hexyl-cAMP for the slime mold PDE. Since hexyl-cAMP is also a good substrate of the enzyme, protection of cAMP is only temporal. Thus pulses of hexyl-cAMP will generate pulses of cAMP, which will lead to the induction of phosphodiesterase and to an acceleration to aggregation competence.

Is it possible to explain the induction of PDE by other cAMP derivatives (Van Haastert *et al.*, 1981) in the same way as with hexyl-cAMP? 3′-NH-cAMP and cAMPS do not inhibit phosphodiesterase activity at 10^{-4} *M* (data not shown), while these compounds induce PDE activity. Furthermore, several derivatives (N^1-oxide-cAMP, 2-bromo-cAMP, and 2′-deoxy-cAMP) induce significant PDE activity at 10^{-7} *M*; at this concentration these compounds are not inhibitory to the enzymes.

These results do not disagree with our original hypothesis based on the close correlation between the cyclic nucleotide specificity of chemotaxis and the specificity of phosphodiesterase induction that the signal for phosphodiesterase induction in *D. discoideum* is detected by the chemotactic receptor.

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